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Upregulated hepatic expression of mitochondrial PEPCK triggers initial gluconeogenic reactions in the HCV-3 patients

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ABSTRACT

Objective: To identify the differential expression of candidate gluconeogenic genes which may initiate hepatitis C virus (HCV) related metabolic disorder during early stages of disease.**Methods:** Patients of diverse age and sex, with positive HCV genotype 3 (HCV-3) RNA in serum and with no history of other related infections, co-infections, alcoholism, diabetes or chemotherapeutic treatments were considered for this study. Semi-quantitative reverse transcriptase PCR analysis and quantitative fold change analysis of the fresh liver biopsies of eight chronically infected HCV-3 patients and six healthy individuals were evaluated for three potential biomarkers involved in glucose homeostasis induction, namely mitochondrial phosphoenolpyruvate carboxykinase 2 (PCK2), glucose-6-phosphatase catalytic subunit (G6PC) and associated forkhead box protein 01 (FOXO1).**Results:** Symptomatic evaluation, clinical history and blood test were conducted according to general disease prognosis procedures and reported here. Significantly upregulated expression of *PCK2* independent of age, sex and viral infectivity levels in all HCV patients was observed, whereas no significant changes in the expression of *G6PC* and *FOXO1* were found.**Conclusions:** *PCK2* triggers initial gluconeogenic reactions which ultimately result in the accumulation of glycogen in the liver hepatocytes. We therefore suggest that the overproduction of *PCK2* has important physiological role in the onset of metabolic disorder in the HCV-3 patients.

1. Introduction

Approximately, 170 to 200 million people are infected with hepatitis C virus (HCV) worldwide, which is about 3.3% of the world's population [1,2]. Infection with HCV is associated with an increased risk of fatty liver disease, diabetes type-2 (DT2) and a condition referred to as non-alcoholic steatohepatitis (NASH) [3]. Fatty liver disease is a state in which abnormal retention of massive vacuoles of triglyceride fat and glycogen accumulates in liver cells through the process of steatosis.

NASH is a chronic liver condition in which both fatty liver disease and DT2 are present but other causes of fat accumulation, such as alcohol abuse, autoimmune hepatitis, alpha-1 antitrypsin deficiency, corticosteroids and estrogens use are absent [4]. In Pakistan, more than 10 million people are assumed to be infected with HCV and the most prevalent genotype is 3 [5]. Among all HCV genotypes, genotype 3 has more significant association with accelerated fibrosis comparatively [6]. Insulin resistance and DT2 can adversely affect HCV infected patients by reducing SVR and accelerate disease progression [3,7]. In addition to that, sustained virological response is one of the major causes of HCV associated steatosis and an increased risk of hepatocellular carcinoma [7,8]. Furthermore, higher incidence of transplant-related morbidity and mortality is observed in patients with insulin resistance and DT2 [9].

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Table 1

History and clinical diagnostic data of all enrolled subjects (HCV+ and HCV– control).

| Sample name | Age (yrs) | Sex | ALT U/L | Viral load (copy/mL) | Symptoms description | Blood glucose levels | Histopathological abnormalities | Abdominal ultrasound |
|-------------|-----------|-----|---------|----------------------|---|----------------------|---------------------------------|----------------------|
| HCP1 | 47 | F | 86 | 290000 | No hepatitis C symptoms and general health issues ^d | Normal ^a | Not-NASH ^b | Normal ^c |
| HCP2 | 32 | M | 79 | 350000 | Few hepatitis C symptoms such as dizziness and body ache ^d | Normal ^a | Not-NASH ^b | Normal ^c |
| HCP3 | 38 | F | 65 | 605000 | No hepatitis C symptoms and general health issues ^d | Normal ^a | Not-NASH ^b | Normal ^c |
| HCP4 | 35 | M | 29 | 84930 | No hepatitis C symptoms and general health issues ^d | Normal ^a | Not-NASH ^b | Normal ^c |
| HCP5 | 29 | M | 69 | 336841 | Few hepatitis C symptoms such as dizziness and weakness ^d | Normal ^a | Not-NASH ^b | Normal ^c |
| HCP6 | 16 | M | 117 | 147000 | No hepatitis C symptoms and general health issues ^d | Normal ^a | Not-NASH ^b | Normal ^c |
| HCP7 | 36 | F | 53 | 220000 | No hepatitis C symptoms and general health issues ^d | Normal ^a | Not-NASH ^b | Normal ^c |
| HCP8 | 45 | F | 62 | 228689 | Few hepatitis C symptoms such as dizziness and body ache ^d | Normal ^a | Not-NASH ^b | Normal ^c |

^a Fasting and post-prandial blood glucose levels <7 mmol/L and <9 mmol/L respectively, were considered as 'Normal'.^b All biopsies were scored <3 as described elsewhere [19] and were diagnosed as not nonalcoholic steatohepatitis or 'not NASH'.^c 'Normal' in parenchymal echotexture with no intra-hepatic or extra-hepatic cholestasis and no focal lesions.^d Detailed description of hepatitis C symptoms is described in the introduction section.

In recent years, the association between HCV infection and gluconeogenesis has been extensively described [10,11]. Microarray analysis of HCV infected subjects (in particular genotype 3) has shown the induced transcription of several metabolic genes in the liver tissues [3,6]. Expression levels of hepatic phosphoenolpyruvate carboxykinase (PCK) which is an important gluconeogenesis enzyme, greatly influences the modulation of glucose synthesis [11]. Mitochondrial PCK is encoded by the *PCK2* gene located at 14q11.2 chromosome in humans. A cytosolic form of this protein is encoded by a different gene, *PCK1*, which has received the most attention due to its higher expression in mouse liver, but in humans both isozymes are expressed approximately equally [12]. G6PC catalyzes the hydrolysis of D-glucose 6-phosphate to D-glucose and orthophosphate and is a key functioning enzyme involved in gluconeogenesis and glycogenolysis. Forkhead box O1 (FOXO1) is a transcription factor responsible for the regulation of *PCK* and *glucose-6-phosphatase, catalytic subunit (G6PC)* gene expression. Mis-regulated expression of hepatic PCK, G6PC and FOXO1 in HCV infected hepatocellular carcinoma cell lines (namely huh7.5 and huh.8) have shown a direct involvement of the HCV core and nonstructural 5A protein [10,11,13,14]. Higher expression levels of *PCK*, *G6PC* and *FOXO1* mRNA in the liver biopsies of HCV patients with steatosis were observed when compared with normal liver hepatocytes [15].

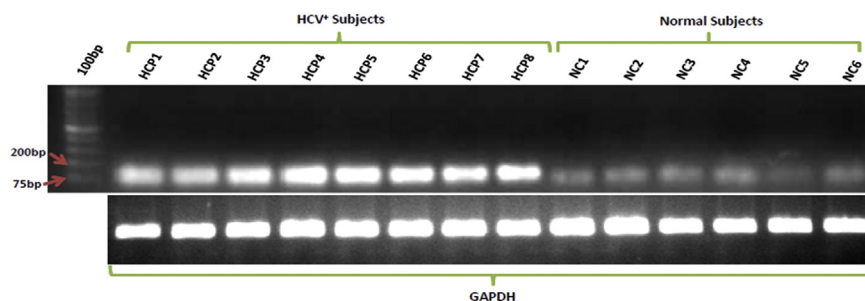
Clinical symptoms of HCV infection such as fatigue, joint pain, belly pain, itchy skin, sore muscles and dark urine, only

develop in minority of cases. This paucity of symptom keeps most cases undiagnosed even after many years of HCV infection [16]. As the association of HCV infection and DT2 is present before the onset of cirrhosis, here we aimed to evaluate the expression of PCK2, G6PC and FOXO1 as potential biomarkers to induce HCV associated glucose homeostasis and associated metabolic liver disorders.

2. Materials and methods

2.1. Patient(s) characteristics

All patients were enrolled for sampling according to the ethical guidelines of the 1975 Declaration of Helsinki after approval of hospital ethic committee. The routine histopathological test was ordered by the patient's physician after explaining the risks and benefits of testing to the patient and had obtained written informed consent. A recent clinic note summarizing the case and the prior workup was also provided by the patient's physician. A short clinical questionnaire was constructed to collect the patients' history by interview. Subjects of varying gender and age were enrolled. As our aim was to look at the expression levels exclusively due to HCV replication, we adopted stringent enrollment criteria and only those patients who met the following conditions were enrolled: 1) chronically infected HCV patients of diverse age, sex (male/female) with positive viral RNA in serum; 2) No other known viral and/or bacterial co-infection, alcoholism

**Figure 1.** Semi-quantitative results of RT-PCR, illustrating relative gene expression pattern of *PCK2* in all HCV+ Subjects vs. HCV– controls.

Lane 1 100 bp ladder, Lane 2, 3, 4, 5, 6, 7, 8, 9 showing 104 bp *PCK2* expression levels of HCP1, HCP2, HCP3, HCP4, HCP6, HCP7 and HCP8 respectively. Lane 10–15 showing 104 bp *PCK2* expression levels of normal control sample NC1 to NC6 (Table 2). All samples were normalized with *GAPDH* as endogenous control.

Table 2

Detailed specifications of oligos.

| Srl. No. | Target gene name and accession number | Primer sequence 5'-3' | Amplicon size |
|----------|--|---|---------------|
| 1 | <i>Homo sapiens FOXO1</i> mRNA NM_002015 | Left-CGCCCTCGAACTAGCTCAAA Right-GCGGGTACACCATAGAATGCA | 109 bp |
| 2 | <i>Homo sapiens PCK2</i> mRNA NM_001018073 | Left-CAAGACCAACCTGGCTATGATG' Right-GGAGTCGACCTTCACTGTCAAAC | 104 bp |
| 3 | <i>Homo sapiens G6PC</i> mRNA NM_000151 | Left-AGTTGTTGCTGGAGTCTGTCA Right-GGTCTACACCCAGTCCCTTGAG3' | 161 bp |
| 4 | <i>Homo sapiens GAPDH</i> mRNA NM_002046 | Left-AGGGGCCATCCACAGTCTTC Right-AGAAGGCTGGGGCTCATTTC | 258 bp |

or diabetes. 3) Patients without any drug or chemical treatment and/or interferon, pegylated interferon and ribavirin therapy treatment history. Postmortem liver samples of healthy individuals with negative HCV viral RNA (RT-PCR) and ≤ 40 alanine transaminase (ALT) levels with no apparent liver abnormalities were taken as HCV negative controls (First Choice[®] Human Tissue Total RNA Ambion[®], Life Technologies[™] USA). Blood tests such as HCV viral load (copies/mL) and HCV genotyping were conducted at the National University of Science and Technology's molecular diagnostic facility as recommended by center for disease control USA (<http://www.cdc.gov/hepatitis/HCV/GuidelinesC.htm>).

2.2. Sample collection

The sampling was done at PIMS Islamabad, following approval by the Hospital Ethics Committee. Written informed consent was obtained from the patients for publication of this report. Two milliliter whole blood samples were collected in a sterile blood collection tube for histological evaluation and partly for RNA extraction. The surgically removed biopsy samples were processed for routine histopathological examination. The sample part unnecessary for histological diagnosis was placed in RNeasy[®] (Ambion[®], Life Technologies[™] USA) for gene expression studies. The negative control sample was prepared from the tissues that had been frozen and stored in RNeasy[®]. All samples were placed at -80°C .

2.3. Clinical diagnostics

Blood samples from each subject were used to conduct clinical diagnostic assessments including complete blood picture, blood glucose and ALT levels, HCV Genotyping and viral load quantification (Table 1). Each patient was examined for histopathological abnormalities and by abdominal ultrasound for visible change in liver echotexture.

2.4. RNA preparation

Total RNA of all tissue samples was extracted by using RNeasy[®]-4PCR Kit (Ambion[®], Life Technologies[™] USA) according to manufacturer's instructions. Total RNA prepared from normal liver tissues that had been frozen/stored in RNeasy[®] before extraction were used as an HCV negative control. Final concentrations of all the RNA samples including HCV negative healthy control were adjusted to 50 ng/ μL by using 260/280-nm absorbance ratio (BioPhotometer Plus & Hellma[®] Tray Cell, Eppendorf).

2.5. Reverse transcriptase quantitative real time polymerase chain reaction (qRT-PCR)

Primers for PCK2, G6PC and FOXO1 were designed with the assistance of the Primer Express 3.0 software (Applied

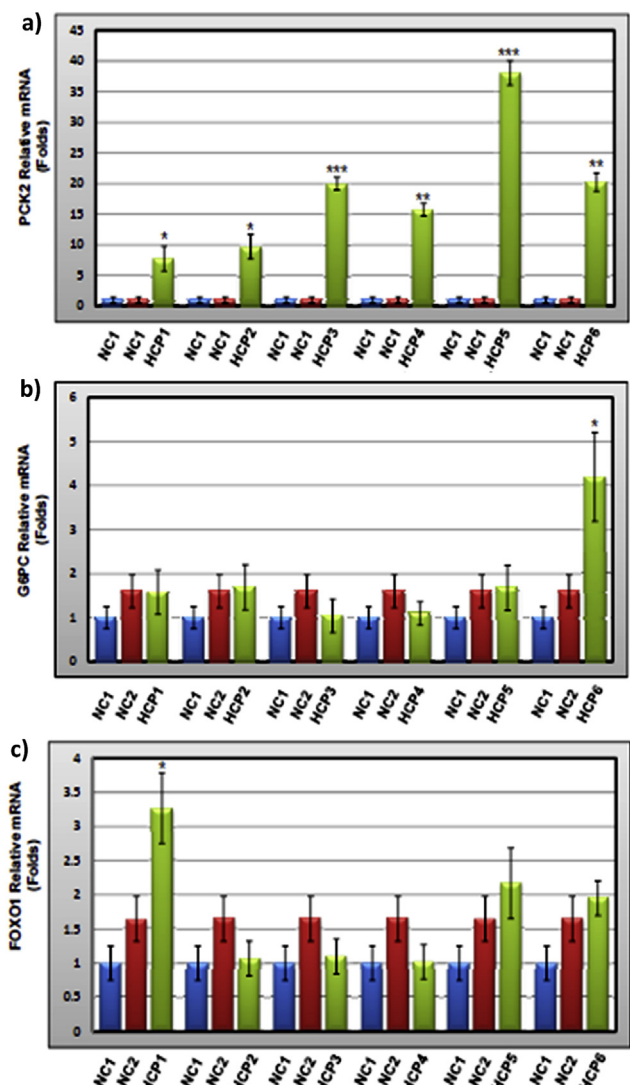


Figure 2. Quantitative RT-PCR analysis of *PCK2*, *G6PC*, and *FOXO1* relative fold changes in the gene expression of individual HCV+ subject were calculated using $\Delta\Delta\text{Ct}$ method.

All Ct (cycle threshold) values were normalized to an endogenous reference gene (*GAPDH*) and shown after log transformation. (2a) *PCK2* (2b) *G6PC* and (2c) *FOXO1* relative to gene expression in HCV negative control subjects is shown in each graph. Error bars indicate S.E.M. *P < 0.05, **P < 0.001, ***P < 0.0001.

Biosystems, USA). First strand cDNA was synthesized by RevertAid™ First Strand cDNA Synthesis Kit by using random hexamer primers (All from Fermentas) were then amplified by using oligos targeting transcripts of *PCK2*, *G6PC* and *FOXO1*. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used with all cDNA samples as an endogenous control.

qRT-PCR of first stand cDNA of three replicates of each sample targeting transcripts of *PCK2*, *G6PC* and *FOXO1* were performed on 7300 Fast Real-Time PCR Systems (Applied Biosystems USA). 50 µL reactions with *Taq* Polymerase with optimal 5× *Taq* buffers, 2 mM dNTPs mix and 25 mM MgCl₂ (All from Fermentas) were prepared. Forty cycles of amplification at 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing) followed by 72 °C for 60 s (extension) were used to measure the fluorescence signal. Reaction concentrations and conditions were adjusted according to the manual instructions of Platinum® SYBR® Green QPCR Super Mix UDG (Invitrogen, U.S.A). Equal quantities of cDNA from normal patients were taken as a negative control. All samples were normalized with reference genes (*GAPDH*). Five microliter PCR products along with control samples and 100 bp ladder (Fermentas) were electrophoresed in ethidium bromide stained 2% agarose gel and resolved using a gel documentation system (Wealtec Dolphin USA). *GAPDH* PCR products were also electrophoresed to determine PCR efficiency.

2.6. Data analysis

Quantitative analyses of the data were carried out using 7300 system SDS software v1.4 (Applied Biosystems, USA) and >35 cycle threshold (Ct) value, out of 40 cycles of amplification were considered as un-detected [17]. Fold difference in gene expression was calculated by using a comparative method ($\Delta\Delta C_t$ method) [18]. Statistical differences (mean ΔC_t values, SDV and SEM) were analyzed using Microsoft Excel. SEM is indicated in error bars. The descriptive statistical data analysis

was calculated using Microsoft Excel and *P* values were calculated using two tailed Student's *t*-test for statistical significance.

3. Results

3.1. Patients' details

Patients' details and reports of all clinical tests conducted for the study are summarized in Table 1.

3.2. Hepatic expression of *PCK2*, *G6PC* and *FOXO1*

Our semi-quantitative analysis showed dramatic upregulation of *PCK2* transcriptional expression in eight chronically infected HCV genotype 3 (CHV3) subjects in comparison with six normal controls but no noticeable or consistent differences were observed between both groups for *G6PC* and *FOXO1* expression (Figure 1 & Table 2 for amplicon size). The band intensity analysis revealed that, although these expression levels are significantly different between both groups, they also vary among CHV3 patients. To describe the risk intensity of developing DT2 in individual patients, the differences in expression were further quantified with qPCR in separate experiments (Figure 1). Due to low RNA yield from liver biopsies we could not precede with two CHV3 patients (HCP7 and HCP8) for further fold change analysis.

Six CHV3 samples were evaluated with quantitative fold change analysis and compared with two normal controls. *GAPDH* normalized expression of *PCK2*, *G6PC* and *FOXO1* in HCP1 was ~7.78, ~1.58 and ~3.27 fold, respectively. For HCP2 *GAPDH* normalized expression of *PCK2*, *G6PC* and *FOXO1* was ~9.71, ~1.69 and ~1.07 fold, respectively (Figure 2). For HCP3 the *GAPDH* normalized expression of *PCK2*, *G6PC* and *FOXO1* was ~15.77, ~1.04 and ~1.1 folds, respectively. For HCP4 the *GAPDH* normalized expression of *PCK2*, *G6PC* and

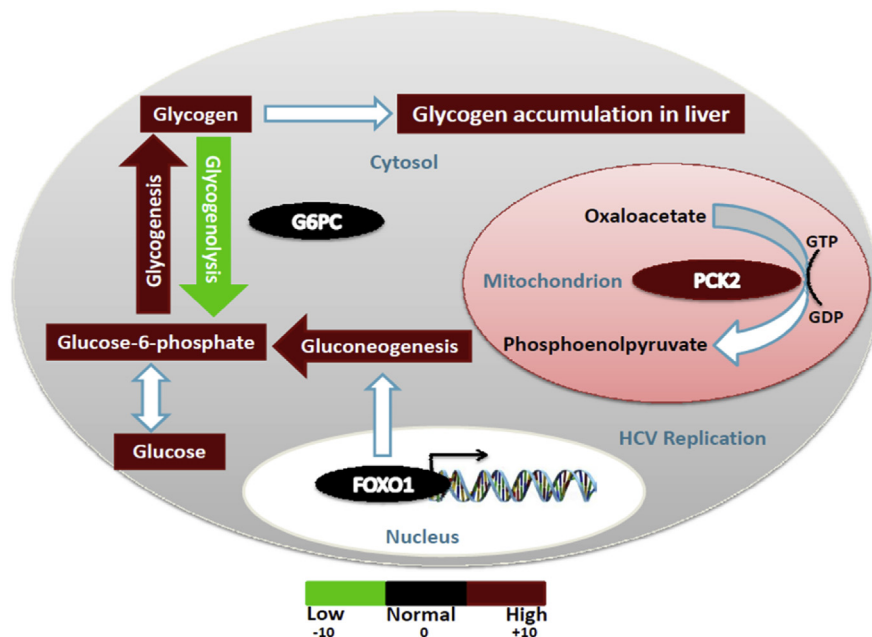


Figure 3. Model representation of initial stages of fatty liver disease and hyperglycemia development in the HCV infected liver hepatic cells. Color codes representing low (green), normal (black) and high (red) expression of metabolic genes of enzymes and transcription factor (*PCK2*, *G6PC* and *FOXO1*) involved in the important pathways of glucose metabolism. Changes in these gene expressions may result in the dysregulated glucose metabolic pathways in the liver hepatocytes which may lead to metabolic disorder in the later stages of the disease.

FOXO1 was ~20, ~1.1, ~1.02 fold, respectively. For HCP5 the *GAPDH* normalized expression of *PCK2*, *G6PC* and *FOXO1* was ~38.05, ~1.68, ~2.17 fold, respectively and for HCP6 the *GAPDH* normalized expression of *PCK2*, *G6PC* and *FOXO1* was ~20.24, ~4.19, ~1.95 fold respectively (Figure 2). Based on these results we have suggested a hypothetical model of glycogen accumulation in the liver which ultimately results in the development of fatty liver disease in the HCV infected patients (Figure 3).

To exclude the possible effects of other factors such as gender, HCV viral load and age in the development of glucose homeostasis, correlation analysis of *PCK2*, *G6PC* and *FOXO1* gene expression was done. No correlations were found among HCV infected patients, between altered gene expression of *PCK2*, *FOXO1* and *G6PC* with gender and HCV viral load (Data not shown).

4. Discussion

HCV nonstructural proteins interact with gluconeogenic enzymes (PCK, G6Pases) and associated transcription factors (FOXO1) and dramatically affect their gene expression regulation in HCV infected hepatocellular carcinoma cells such as Huh 7.5, Huh.8. The dominant effect of FOXO1 proteins among other transcriptional factors and cofactors such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha, cAMP response element-binding and CCAAT enhancer-binding proteins has recently been reported [10,11,13,14]. To our knowledge, this is the first study highlighting upregulated mitochondrial PCK as an early biomarker of metabolic disorder in the HCV-3 patients. In the normal human liver PCK2 express with equal proportion as cytosolic PCK isozyme, PCK1 [19]. The mitochondrial location PCK2 is crucial for initial gluconeogenic reactions as the carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase occurs in the mitochondrion. For cytosolic PCK, oxaloacetate needs to be shuttled out, but for mitochondrial PCK, no shuttle of oxaloacetate is needed [4,19]. Furthermore FOXO1 is a well known transfection factor which induces expression G6Pases and PCK1 [13] but its role in the regulation of PCK2 was unknown. In our results, we found upregulated expression PCK2 but on the other hand expression of FOXO1 was similar in HCV patient as compared to normal control. This therefore suggests that FOXO1 is not involved in the expression of PCK2. Normal expression of G6PC also confirms that PCK2 mediate gluconeogenic reactions by independent mechanisms.

In our results we observed a dramatic change in the expression of PCK2 consistently on HCV infected patients. Whereas in the case of FOXO1 and G6Pase, this dramatic change of >3 and 4 folds was only observed in HCP1 and HCP6, respectively. All other HCV patients showed almost similar expression of FOXO1 and G6PCas that of normal subjects. As the upregulated expression levels of FOXO1 and G6PC were reported in the Huh. Cells which represent hepatic steatosis or hepatocellular carcinoma, here we assume that these changes in the expression may only appear in the later stage of the disease. Moreover, HCP6 has unusual liver damage as indicated by his abnormally high ALT levels (117 U/L) which may also have some relation to the elevated expression of G6PC. This may also be true for HCP1 (ALT 86 U/L). On the other hand, PCK2 showed a consistently high expression level ranging from ~7 to 38 folds,

independent of any other factor such as age, gender, HCV or ALT. Therefore, we suggest that PCK2 triggers initial gluconeogenic reactions which result in the accumulation of glycogen in the liver hepatocytes. Furthermore, functional effect of PCK2 must be considered in the future studies to study the overall physiology of metabolic disorder associated with HCV infection.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

- [1] Global surveillance and control of hepatitis C. Report of a WHO consultation organization in collaboration with the Viral Hepatitis Prevention Board. *J Viral Hepat* 1999; **6**: 35-47.
- [2] Tran G. The role of hepatitis C virus in the pathogenesis of hepatocellular carcinoma. *Biosci Horizons* 2008; **1**: 167-175.
- [3] Zekry A, McHutchison JG, Diehl AM. Insulin resistance and steatosis in hepatitis C virus infection. *Gut* 2005; **54**: 903-906.
- [4] Berg JM, Tymoczko JL, Stryer L. *Biochemistry*. New York: W. H. Freeman and Company; 2002.
- [5] Waheed Y, Shafi T, Safi SZ, Qadri I. Hepatitis C virus in Pakistan: a systematic review of prevalence, genotypes and risk factors. *World J Gastroenterol* 2009; **15**: 5647-5653.
- [6] Bochud PY, Cai T, Overbeck K, Bochud M, Dufour DF, Müllhaupt B, et al. Genotype 3 is associated with accelerated fibrosis progression in chronic hepatitis C. *J Hepatol* 2009; **51**: 655-666.
- [7] Pattullo V, Heathcote J. Hepatitis C and diabetes: one treatment for two diseases? *Liver Int* 2010; **30**: 356-364.
- [8] Fartoux L, Poujol-Robert A, Guécho J, Wendum D, Poupon R, Serfaty L. Insulin resistance is a cause of steatosis and fibrosis progression in chronic hepatitis C. *Gut* 2005; **54**: 1003-1008.
- [9] Giordanino C, Ceretto S, Bo S, Smedile A, Ciancio A, Bugianesi E, et al. Type 2 diabetes mellitus and chronic hepatitis C: which is worse? Results of a long-term retrospective cohort study. *Dig Liver Dis* 2012; **44**: 406-412.
- [10] Choudhury M, Qadri I, Rahman SM, Gloeckler JS, Janssen RC, Friedman JE. C/EBP β is AMP kinase sensitive and up-regulates PEPCK in response to ER stress in hepatomacells. *Mol Cell Endocrinol* 2012; **331**: 102-108.
- [11] Qadri I, Chouradry M, Rahman SM, Knotts TA, Janssen RC, Iwahashi M. Insulin resistance, steatosis, and increased PEPCK gene expression during hepatitis C virus (HCV) subgenome replication: role of NS5A and C/EBP β . *J Biol Chem* 2012; **287**: 37340-37351.
- [12] Beale EG, Harvey BJ, Forest C. PCK1 and PCK2 as candidate diabetes and obesity genes. *Cell Biochem Biophys* 2007; **48**: 89-95.
- [13] Banerjee A, Meyer K, Mazumdar B, Ray RB, Ray R. Hepatitis C virus differentially modulates activation of forkhead transcription factors and insulin-induced metabolic gene expression. *J Virol* 2010; **84**: 5936-5946.
- [14] Deng L, Shoji I, Ogawa W, Kaneda S, Soga T, Jiang DP, et al. Hepatitis C virus infection promotes hepatic gluconeogenesis through an NS5A-mediated, FoxO1-dependent pathway. *J Virol* 2011; **85**: 8556-8568.

- [15] Valenti L, Rametta R, Dongiovanni P, Maggioni M, Fracanzani AL, Zappa M, et al. Increased expression and activity of the transcription factor FOXO1 in non-alcoholic steatohepatitis. *Diabetes* 2008; **57**: 1355-1362.
- [16] Shiffman ML. *Chronic hepatitis C virus advances in treatment, promise for the future*. New York: Springer-Verlag; 2011.
- [17] Sheikh TI, Qadri I. Expression of EBV encoded viral RNA 1, 2 and anti-inflammatory cytokine (interleukin-10) in FFPE lymphoma specimens: a preliminary study for diagnostic implication in Pakistan. *Diagn Pathol* 2011; **6**: 70.
- [18] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2011; **25**: 402-408.
- [19] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al., Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321.